PHOSPHATASES OF MARINE ALGAE[1]

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The phosphatases are enzymes important to life because of their ability to hydrolyze phosphate esters or to transfer phosphate from one organic group to another. Phosphomonoesterases hydrolyze monoesters of phosphoric acid,

$$RO - PO_3H_2 + H_2O \rightarrow ROH + H_3PO_4,$$

and are classified as acid- or alkaline-phosphatases depending on the pH at which maximum activity occurs. Other phosphatases hydrolyze diesters of phosphoric acid, pyrophosphates, or metaphosphates; unless otherwise stated, however, the term "phosphatase" will hereafter be used to designate only phosphomonoesterases. Constitutive enzymes are those always formed by cells, regardless of the medium in which they are grown. Induced enzymes are those produced by an organism only when it is exposed to a specific substrate; it then permits the organism to utilize this substrate. Repressible enzymes are those synthesized only when the concentration of a particular substance, a represser, becomes very low.

Phosphatases in algae have been reported by several workers recently. Brandes and Elston (1956), using histochemistry and electron micrography, reported the alkaline phosphatase of *Chlorella vulgaris* to be localized at the cell surface. Talpasayi (1962), studying the inhibition of acid phosphatases of algae by molybdenum, found that living cell suspensions showed more enzyme activity than broken cells. Eppley (1962) found that several species of intertidal algae hydrolyze pyrophosphate and assimilate a portion of it; most of the resulting orthophosphate remained in the medium, however. Galloway and Krauss (1963) found that pyrophosphate induced the production of pyrophosphatase in *Chlorella pyrenoidosa* cultures; cell walls from disrupted *Chlorella* contained most of this enzyme. Overbeck (1962) found that *Chlorella pyrenoidosa* cultures split pyrophosphate and glycerophosphate. He also reported that *Scenedesmus quadricauda* contained three phosphomonoesterases and three pyrophosphatases but they seemed to be inside the cell rather than at the surface and the substrates did not penetrate the cell wall. Living *Scenedesmus* did not assimilate pyrophosphate or phosphomonoesters rapidly even after seven weeks in the presence of these substrates. Price (1962) showed that 2 m*M* phosphate was sufficient to repress synthesis of the acid phosphatase of *Euglena gracilis*.

Many species of algae are capable of obtaining phosphorus from esters in order to sustain growth in the absence of orthophosphate (see review by Provasoli, 1958). We found that many species of marine algae produce alkaline phosphatase when they

[1] Contribution No. 1526 from the Woods Hole Oceanographic Institution.

[2] Supported by U. S. Atomic Energy Commission Contracts AT(30-1)-1918 and AT(30-1)-3145.

become phosphorus-deficient. This enzyme hydrolyzed dissolved phosphate esters on or outside the cell; the cell then absorbed only the phosphate ion, leaving the organic moiety in the medium.

We are very grateful to Drs. R. R. L. Guillard, J. C. Hellebust, and C. A. Price for many stimulating discussions and suggestions. Dr. Guillard also generously furnished the pure cultures of marine algae; the *Chlamydomonas reinhardi* culture was obtained from the Harvard University laboratory of Dr. R. P. Levine through the courtesy of Miss J. C. Brungard. We are also grateful to Drs. J. H. Ryther, R. R. L. Guillard, and A. Torriani for reading and criticizing the manuscript.

Methods

Stock axenic algal cultures were maintained in half-strength Medium *f* (Guillard and Ryther, 1962): 75 mg. $NaNO_3$, 5 mg. $NaH_2PO_4 \cdot H_2O$, 5 mg. FeEDTA, 15–30 mg. $Na_2SiO_3 \cdot 9H_2O$, 100 μg. thiamine·HCl, 0.5 μg. Biotin, 0.5 μg. vitamin B_{12}, 10 μg. $CuSO_4 \cdot 5H_2O$, 22 μg. $ZnSO_4 \cdot 7H_2O$, 10 μg. $CoCl_2 \cdot 6H_2O$, 180 μg. $MnCl_2 \cdot 4H_2O$, 6.3 μg. $Na_2MoO_4 \cdot 2H_2O$, 1 liter filtered sea water. *Chlamydomonas* spp. were furnished with NH_4Cl instead of $NaNO_3$ as the nitrogen source. Stock cultures were tested periodically for bacterial contamination by inoculating into sea water medium containing 0.1% tryptone. Phosphorus-deficient algae were produced by growing them in sea water medium containing double the concentration of all the above nutrients except phosphorus; it was reduced to 7 μM or less. All cultures were grown at 20° C. except *Detonula confervacea* and *Thalassiosira nordenskioldii* which were grown at 5° C. Cells were counted using a Spencer "Bright Line" or a Palmer counting chamber. Cell dimensions were obtained with a calibrated ocular micrometer and cell volumes were calculated by formulae for similar geometric solids (sphere, cylinder, etc.).

Cellular protein was measured by the Folin-Ciocalteu phenol reagent by a method that we modified from Lowry *et al.* (1951). Algal suspensions were centrifuged for 5 minutes and the supernatant was discarded. The cells were resuspended in 5 ml. of 10% trichloroacetic acid (TCA) and held 10 minutes at 100° C. to precipitate protein and dissolve nucleic acids and other acid-soluble material. The precipitate was centrifuged and washed twice with 5 ml. of 10% TCA. The protein was redissolved in 0.5 ml. 1 N NaOH overnight, then 5 ml. of Lowry's Reagent D (50 parts 2% Na_2CO_3 plus 1 part 0.5% $CuSO_4 \cdot 5H_2O$ in 1% sodium-potassium tartrate; mixed fresh daily) was added and the volume was made up to 6 ml. with distilled water. After 10 minutes 0.5 ml. 1 N Folin-Ciocalteu phenol reagent was added and the absorbance at 750 mμ was read 30 minutes later on a Beckman DU spectrophotometer. Protein standardization was performed on bovine serum albumin by the Lowry *et al.* (1951) procedure.

Enzyme activity was assayed by the rate at which nitrophenol accumulated during hydrolysis of *p*-nitrophenyl phosphate (NPP) at known hydrogen ion concentrations. The NPP stock consisted of 0.1 g. of NPP plus 2.5 g. $MgSO_4$ in 50 ml. distilled water. The pH range 3.0 to 6.8 was covered by 1 M citric acid-potassium citrate solutions and the range 7.2 to 9.8 by 1 M Tris (hydroxymethyl)amino methane·HCl solutions. The actual pH value of filtered sea water

containing these buffer solutions was measured with a Beckman Model 76 hydrogen ion meter. For each analysis, the algal culture : NTP stock : citrate buffer stock : autoclaved-sea water diluent ratios were usually about 1:1:1:30. In order to obtain adequate alkaline buffering however, it was necessary to use twice as much Tris, giving 1:1:2:30 ratios. Unless otherwise noted, all measurements of enzyme activity were performed on whole, living cells in darkness or dim light at 19°–21° C. After incubation, sufficient pH 10 Tris buffer was added to the low pH samples to bring out nitrophenol color (pH 9) and the absorbance of nitrophenol was measured in a Beckman DU spectrophotometer at 410 mμ in 1-cm. or 10-cm. cells. Enzyme activity was calculated from the nitrophenol-time regression; one "Unit" represents the amount of enzyme that causes a change in optical density of 0.001 per minute in a 1-cm. cell.

The rate of assimilation of phosphorus by algae from glucose-6-phosphate (G-6-P), adenosine monophosphate (AMP), and α-glycerophosphate (α-GP) was measured by periodically determining the phosphorus content of the algal cells. Two ml. of cultures of phosphorus-deficient cells were added to 8 ml. of filtered, autoclaved sea water (pH ~ 8) containing 0.1–0.2 μmole of one of these three esters in centrifuge tubes. Periodically, duplicate tubes were centrifuged for 5 minutes, the supernatant was discarded, and the cellular phosphorus was determined by measuring the orthophosphate after hydrolysis in 0.55 N H_2SO_4 at 140° C. for 5 hours (Ketchum *et al.*, 1955). Total phosphorus initially present in the sea water, the algal cultures, and the three substrates was measured the same way. Inorganic phosphate of sea water was measured by the method of Wooster and Rakestraw (1951).

Rate of uptake of the glucose from G-6-P was determined from the decrease in radioactivity of a C^{14}-labeled solution. Ten μc. of the Ba salt of D-glucose-1-C^{14}-6-phosphate were dissolved in 3 ml. distilled water and converted to the Na salt by passage through a 7 × 30-mm. strong cation exchange column (Dowex 50) in the Na^+ form; the column was rinsed with 2 ml. of distilled water. This was diluted 1:100 to obtain a suitable count-rate and 0.25 ml. was added to centrifuge tubes containing autoclaved sea water (pH ~ 8), G-6-P carrier, and phosphorus-deficient algae as described above. Subsamples of this suspension were periodically centrifuged and samples of the supernatant were dispensed (0.2–0.5 ml) onto planchets, dried, and the C^{14} counted with a windowless gas flow detector. The same volumes of the original whole suspension were also dried, and the initial count rate thus obtained had geometry and self-absorption factors similar to the supernatant samples.

Results

Phosphatase synthesis by deficient algae

Alkaline phosphatase is produced abundantly when algae are grown in a phosphate-limited medium, or stated conversely, its production is repressed in phosphorus-sufficient algae (Table I). In each species the enzyme activity of P-deficient cells was at least 30 times greater than in cells limited by vitamins, iron and trace metals, nitrogen, or silicon. (*Phacodactylum* has no requirement for vitamins or silicon, nor *Coccolithus* for silicon; hence these experiments were not performed.)

TABLE 1

Effect of nutrient limitation on production of alkaline phosphatase. Cultures were grown in Medium f deficient in one of the 5 types of nutrients until cell numbers increased less than 10% per day. Enzyme activity (Units/10^5 cells) was measured at 20° 25° C. All measurements were made at pH 8.0, although later it was found that this was not at the pH optimum for the phosphatase of these species.

Nutrients Omitted:	Thiamin Biotin, B_{12}	Fe, Cu, Zn, Co, Mn, Mo	N	Si	P
Phaeodactylum tricornutum	—	1.1	2.1	—	62
Cyclotella nana (clone 13-1)	0	0.03	0.10	0.03	6.3
C. nana (clone 3H)	0	0.17	0.02	0.21	12
Skeletonema costatum	0.04	0.27	0.27	0.13	12
Coccolithus huxleyi	0.05	0.04	0	—	59

The low enzyme activities in P-sufficient cultures are not the result of inhibition of enzyme activity by phosphate in the medium. In one experiment we measured the phosphatase activity of *Coccolithus huxleyi* in the presence of various orthophosphate concentrations and found a nearly linear inhibition of activity amounting to 80% at 1.0 m*M*. However, the initial phosphate concentration of Medium *f* is

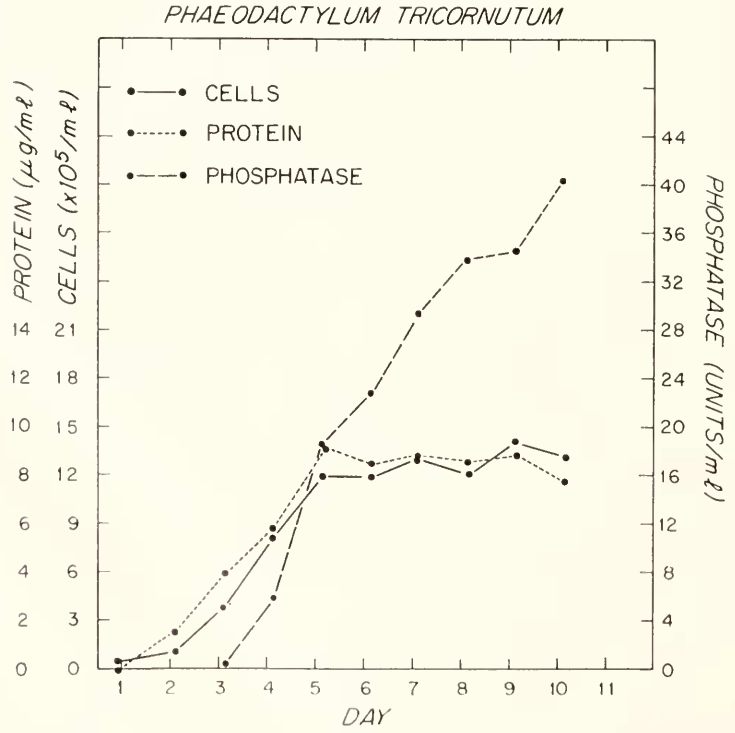

FIGURE 1. Changes in cell numbers, protein content, and enzyme activity during growth of a *Phaeodactylum tricornutum* culture; 15 liters stirred by constant aeration; 2.5 μ*M* P; 25° C.; 300 f.c. illumination; enzyme measured at pH 9.7.

only 72 μM and the inhibition of enzyme activity should have only been about 6% even if no phosphate had been assimilated by the cells. As discussed below, the amount of phosphatase in a culture increases with time; thus, comparisons of the values for the different species in Table I are unwarrantable.

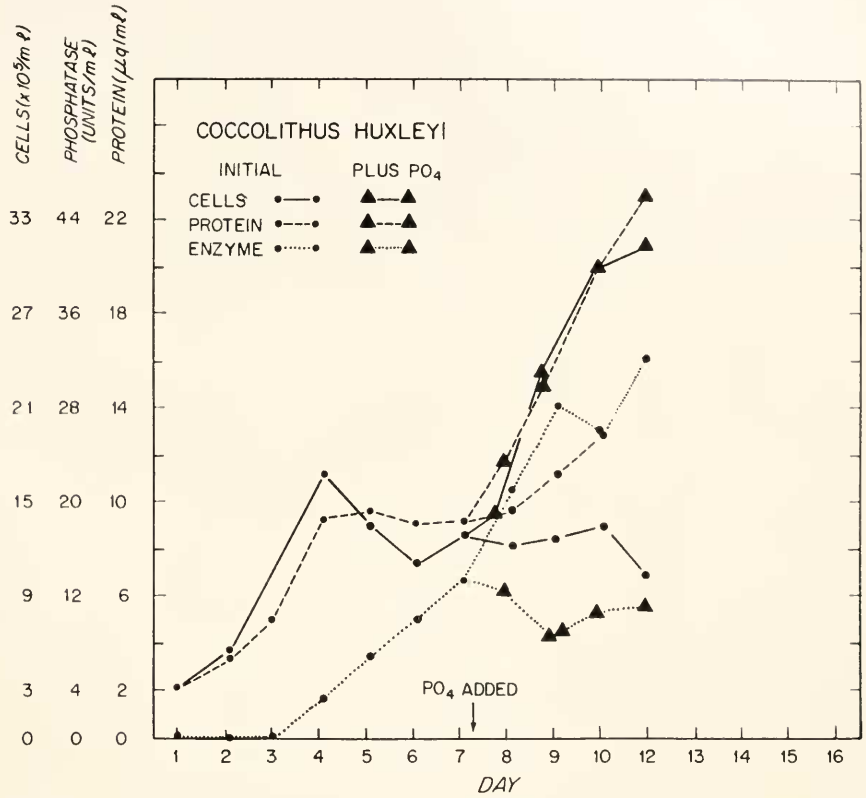

FIGURE 2. Changes in cell numbers, protein content, and enzyme activity during growth of a *Coccolithus huxleyi* culture: 1 liter shaken several times daily; 3.7 μM P; 20° C.; 300 f.c. illumination; enzyme measured at pH 9.7; phosphate added on day 7 increased the content of the medium by 25 μM P.

Time course of phosphatase production

The production of alkaline phosphatase in *Phaeodactylum* began when the average cell still had enough phosphorus to divide at least once more, *i.e.*, about two days before cell division and protein synthesis stopped (Fig. 1). Enzyme production continued at a nearly linear rate at least 5 days after the apparent halting of net protein production.

The same pattern of initiation of enzyme production just before growth stopped was repeated in two experiments with *Coccolithus*, the results of one being shown in Figure 2. Repression of enzyme synthesis by phosphate was confirmed when this nutrient was added to half of the culture on day 7, the remaining half representing

a control. Phosphate stopped enzyme production whereas cell numbers and cellular protein increased again at rates comparable to those on days 1 to 3.

The cellular phosphorus per unit volume of P-deficient marine algae ranged from 1 to 15 $\times$ 10^{-17} mole P/μ^3 in species of different sizes (Table II). Cellular protein per unit volume, however, differed by less than a factor of 5 among these same species. Some variability in both P- and protein-content might arise from calculation of cell volume, due to irregularity of cell shape and uncertainty as to the thickness of the enveloping frustules, coccoliths, or cell walls. A large vacuole would also tend to lower the amount of P or protein per unit whole cell volume. The P:Protein ratios calculated from Table II were more nearly constant, from 230

TABLE II

Cell volume, phosphorus, protein, and maximum observed rate of production of phosphatase in phosphorus-deficient marine algae

	Cell volume (μ^3 cell)	Cell P (10^{-17}mole/μ^3)	Cell protein (10^{-14} g μ^3)	Enzyme production rate (10^{-8} units μ^3·day)
Chrysophyceae				
Isochrysis galbana	115	1.0	4.3	6
Cricosphaera carterae	1240	1.7	5.8	16
Coccolithus huxleyi	73	2.2	6.2	26
Bacillariophyceae				
Phaeodactylum tricornutum	53	3.2	5.7	10
Chaetoceros simplex	120	1.6	5.9	6
Thalassiosira fluviatilis	1300	2.9	7.4	10
Cyclotella caspia	210	3.5	7.6	6
C. nana (clone 13-1)	140	3.4	10	0.2
C. nana (clone 3-H)	85	2.7	7.8	2
C. cryptica	640	4.3	15	11
Nitzschia closterium	84	15	19	3
Skeletonema costatum	160	8.0	12	0.5
Dinophyceae				
Amphidinium carteri	660	2.9	10	0.4

to 790 μmole P/g. protein. Some algae synthesized alkaline phosphatase much faster than others when compared on the basis of cellular volume (Table II). Further work will undoubtedly show conditions under which higher rates occur in some of these algae, but repeated experiments indicate that, under our conditions, there are obvious differences. *Coccolithus*, *Cricosphaera*, *Phaeodactylum*, and *T. fluviatilis* repeatedly showed rapid enzyme production when phosphorus-deficient.

Optimum pH

The rate at which phosphorus-deficient algae hydrolyzed NPP was markedly pH-dependent. The graphs of five representative species (Fig. 3) show that significant enzyme activity generally extends one or more pH units on either side of the peak. Some species have more than one peak. It should be remembered that the absolute height of the alkaline-phosphatase peak is a function of both the length of time since the cells became P-deficient and specific physiological factors. For this

reason emphasis is not placed on the amount of enzyme of one species compared to that of others.

Twenty-nine clones (at least 25 species) of marine algae were tested to determine the pH at which their phosphatases were most active (Table III). Two species had maximum rates less than 0.6 Unit/ml. of culture and are not included in the table. All chrysophytes and diatoms (Bacillariophyceae) had an abundant enzyme with activity at pH 8.6 or higher. *Detonula* had a slightly higher peak at pH 6 than at pH 9, but this might have only been the result of its lower metabolic rate

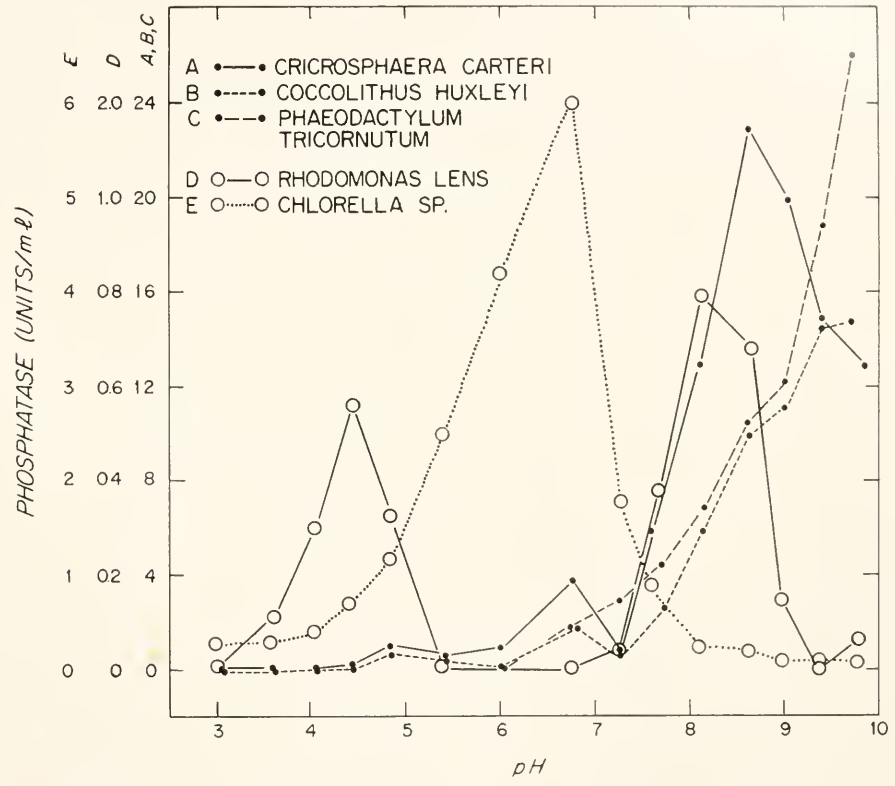

FIGURE 3. Five examples of phosphomonoesterase activity from pH 3 to 10.

at 5° C.; the alkaline phosphatase activity presumably would overtake that of the acid in another few days. *Thalassiosira nordenskioldii*, the only other species grown at 5° C., was also relatively low in alkaline phosphatase, whereas *T. fluviatilis* was similar to other diatoms. The three clones of *Rhodomonas*, although originally isolated from different places, had almost identical pH graphs (see example, Fig. 3) and showed clearly that large amounts of alkaline phosphatase are not produced by *Rhodomonas* under these conditions. Their enzymes are probably constitutive; a repeat experiment with clone F-5A showed essentially the same amount of alkaline phosphatase as the first. The blue-green alga, *Oscillatoria*, produced alkaline phosphatase, whereas *Coccochloris* sp. (clone Syn)

(not shown) did not. *Gymnodinium nelsoni* is interesting because both the acid and the alkaline phosphatases more than doubled during four days of P-deficiency. This is our only example of a phosphorus-repressible acid phosphatase. The two species of *Chlamydomonas* were the only green algae with significant amounts of alkaline phosphatase. Clone 0–5 was extremely rich; furthermore it had two alkaline peaks, the second being 475 Units/ml. at pH 8.6. The chlorophyte, *Dunaliella tertiolecta* (not shown), was tested twice without acid or alkaline phosphatase being detected. *Pyramimonas* was also tested twice. *Chlorella* sp.

TABLE III

The pH optima of algal phosphatases. The clone designation of each of the algae is in parentheses after the name. The habitat type from which clones were obtained and isolated are: E, estuarine or rock pool; N, neritic; and O, oceanic. One Unit/ml. is the enzyme activity per ml. of culture that causes a change in O.D. of 0.001 per minute in a 1-cm. cell.

	pH Optima				
	Acid		Alkaline		Habitat
	Units/ml.	pH	Units/ml.	pH	
Chrysophyceae					
Isochrysis galbana (Iso)	1.9	6.8	28	9.8	E
Monochrysis lutheri (Mono)	—	—	8	9.7	E
Cricosphaera carterae (Cocco II)	3.9	6.8	23	8.6	N
Coccolithus huxleyi (BT-6)	1.8	6.8	15	9.7	O
Bacillariophyceae					
Phaeodactylum tricornutum (Phaeo)	—	—	26	9.7	E
Melosira sp. (T-5)	0.6	6.8	6	9.7	E
Cyclotella nana (3H)	—	—	2.6	9.8	E
C. cryptica (O-3.X)	—	—	37	9.7	E
Detonula confervacea (D. con.)	1.1	6.0	0.7	9.0	E
Thalassiosira fluviatilis (Actin)	0.6	6.8	18	9.8	N
T. nordenskioldii (T. nord.)	—	—	1.3	9.0	N
Nitzschia closterium (N. clost.)	—	—	2.7	9.8	N
Skeletonema costatum (Skel)	—	—	0.7	8.6	N
Chaetoceros sp. (*simplex?*) (BBsm)	2.3	6.8	18	9.7	O
Cyclotella caspia (10-5)	1.5	6.8	12	9.8	O
C. nana (13-1)	—	—	1.5	9.8	O
Cryptophyceae					
Rhodomonas (?) (3C)	0.5	4.4	0.8	8.6	E
Rhodomonas (?) (E-5.X)	0.5	4.4	0.6	8.6	E
Rhodomonas lens (Rhodo)	0.6	4.4	0.8	8.1	O
Cyanophyceae					
Oscillatoria Woronichinii (Sm 24)	0.6	6.8	3.7	9.0	E
Dinophyceae					
Amphidinium carteri (Amphi 1)	—	—	9	9.0	E
Gymnodinium nelsoni (GSBL)	0.8	5.8	0.3	9.8	E
Chlorophyceae					
Chlorella sp. (580)	6	6.8	—	—	E
Stichococcus sp. (GSB Sticho)	2.1	1.8	—	—	E
Pyramimonas sp. (Pyr 2)	0.9	4.8	—	—	E
Chlamydomonas sp. (O-5)	—	—	650	7.6	E
Chlamydomonas sp. (E-17)	—	—	7.5	8.6	E

had more acid phosphatase than any other alga tested, but even this was low relative to the alkaline phosphatases of many other species. Except for *Gymnodinium*, acid phosphatase production did not seem to begin when these algae became P-deficient as was reported in *Euglena gracilis* by Price (1962). The relative constancy in the amount of acid phosphatase within the species tested (Table III) argues that these enzymes are constitutive. It is possible that the alkaline phosphatases found in only small amounts in certain algae are also constitutive; further study of these species is necessary.

The phenomenon of alkaline phosphatase production when phosphate-deficient was found in algae from estuarine, neritic, and oceanic habitats (Table III).

Uptake of phosphate esters

When P-deficient cultures were exposed to three different phosphate esters, all esters were assimilated at the same rate by each algal species (Fig. 4). The intercept at time-zero is the amount of phosphorus in the algae at the beginning of each experiment; the somewhat accentuated uptake during the first 5–8 minutes simply reflects the amount of inorganic phosphate immediately available in the diluting sea water. The curves from 5 to 125 minutes represent uptake of phosphorus from the esters. In *Phaeodactylum* the uptake rate remained almost constant during this time, even though at 124 minutes about 80% of the G-6-P had been assimilated. The initial levels of the other substrates were higher, and such a high proportion was not assimilated during this period. The amount of phosphorus per *Phaeodactylum* cell may be calculated from Figure 4. The final value,

$$\frac{11 \times 10^{-6} \text{ mole P/l.}}{5.7 \times 10^{5} \text{ cells/l.}} = 19 \times 10^{-15} \text{ mole P/cell,}$$

is about 14 times higher than the initial value, but three times less than maximum cellular-P levels reported by Kuenzler and Ketchum (1962) and indicates that these cells were still not saturated. In the other two species not more than half of each substrate was used. The uptake rate decreased noticeably with time in *Cricosphaera*, perhaps indicating that it is more quickly satiated.

The above experiments indicate that, instead of assimilating the whole ester, the algae hydrolyzed the ester extracellularly and took in the phosphate. One might expect the uptake of whole glycerol phosphate, sugar phosphate, or nucleoside phosphate molecules to proceed by different mechanisms and at different rates. However, the remarkable similarity in rates of accumulation of phosphorus from these three esters suggests that the rates were limited by a common mechanism. Alkaline phosphatases of other organisms frequently hydrolyze more than one phosphomonoester (Stadtman, 1961), sometimes at similar rates (*E. coli;* Torriani, 1960). Thus, the enzymatic hydrolysis rate might have limited the rate of phosphorus uptake from these three esters. On the other hand, if sufficient phosphatase were present, the phosphate assimilation rate would be the limiting factor. The phosphorus content of the *Phaeodactylum* inoculated into each of the three esters (Fig. 4) was 1.4×10^{-15} mole/cell. Kuenzler and Ketchum (1962) found the phosphate uptake rate of *Phaeodactylum* in an 8 μM solution to be of the order of 0.32×10^{-15} mole/cell·min. for two hours. These two values give an expected

doubling time of cellular phosphorus of 4.4 minutes; cellular P actually doubled more quickly than this in the initial period (Fig. 4) when the sea water phosphate was being assimilated. The lower rate of P accumulation from the esters than from the sea water phosphate suggests that insufficient phosphatase was present to release phosphate as rapidly as the cells could accumulate it and, therefore, that the ester hydrolysis rate was the limiting factor. The experiments were done at about pH 8 although the phosphatases of these three species are more active at higher pH values (Table III). Here, however, we were interested in the capabilities of algae under approximately normal conditions rather than under conditions optimal for one enzyme but possibly damaging to the whole, living cell.

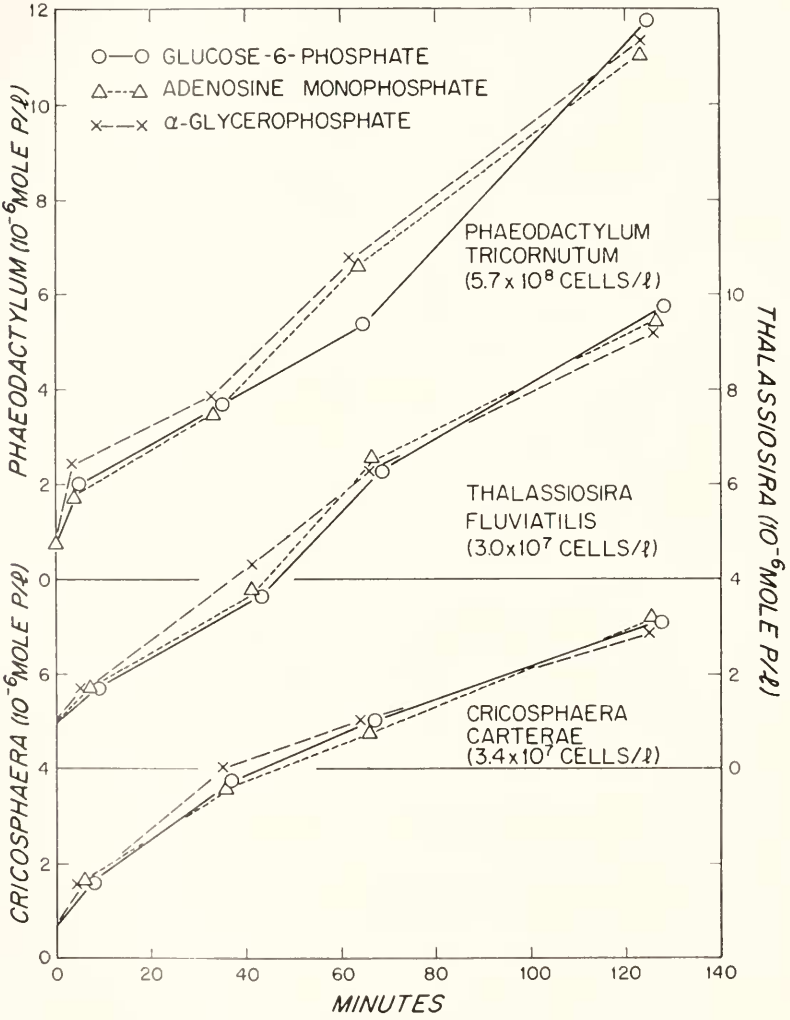

FIGURE 4. Increase in phosphorus content of three species of algae when exposed to three phosphate esters. The initial concentrations of G-6-P, AMP, and α-GP were 12–14, 22 and 14–16 μM P, respectively.

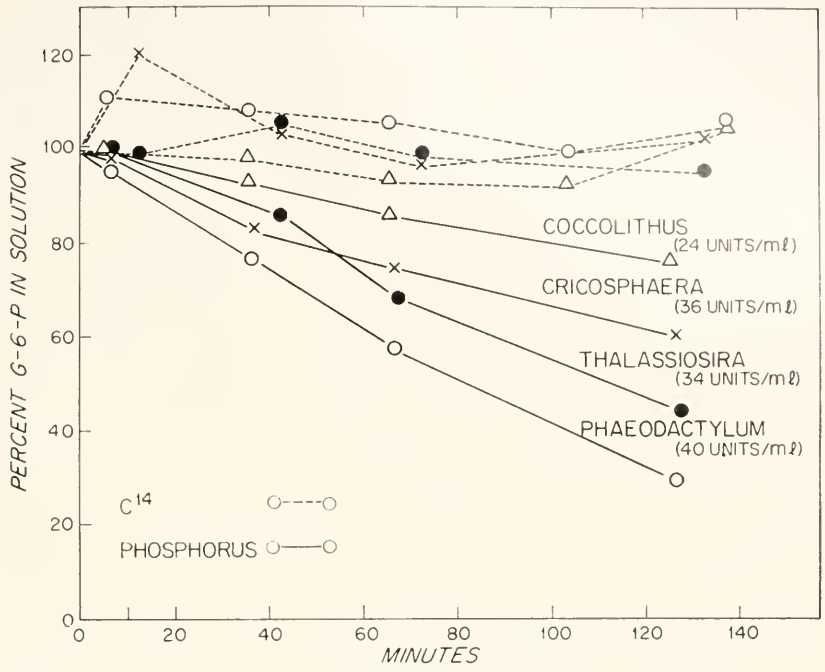

FIGURE 5. The hydrolysis of C^{14}-labeled glucose-6-phosphate by P-deficient *Coccolithus huxleyi, Cricosphaera carteri, Thalassiosira fluviatilis* and *Phaeodactylum tricornutum*. The concentration of the glucose moiety in the medium was determined by measuring C^{14} (dashed lines); the concentration of phosphorus remaining in solution (solid lines) was determined by the difference between the original concentration and the amount measured chemically in the cells. Enzyme activity was measured with NPP at the same time in other portions of each culture; results are shown in parentheses (Units/ml. of culture).

The following experiment showed that only the phosphate is assimilated and that the organic part of the ester remains in solution. When C^{14}-labeled G-6-P was mixed with four cultures of P-deficient algae, radioactive carbon remained in solution while the phosphate was assimilated by the cells (Fig. 5). (The variability in the C^{14} counts was probably caused by self-absorption and geometry variability.) The simplest explanation is that the phosphate was hydrolyzed extracellularly by phosphatase and taken in, whereas the glucose never entered the cell at all. This is substantiated by the general correspondence between the slopes representing the decline of phosphorus in the medium and the phosphatase activity as measured independently with *p*-nitrophenyl phosphate.

Location of the enzyme

The alkaline phosphatase of the two species studied more carefully, *Coccolithus huxleyi* and *Phaeodactylum tricornutum*, is firmly attached rather than being in solution in the cell. Repeated attempts on one or the other species, using sonication, explosive decompression, osmotic shock, freezing and thawing, heating, or treatment with lipase, cellulase, iso-butanol, and various salt and buffer solutions, failed to solubilize the enzyme.

Rothstein (1954) discussed several criteria that might indicate enzyme attachment to a cell surface rather than to an internal organelle, and we have applied some of these criteria to our own data. If the enzyme is located at the cell surface, disrupted cells should show no more activity than whole cells and the pH responses of disrupted and whole cells should be the same. When we sonicated, then centrifuged, cultures of *Coccolithus huxleyi* and *Chlamydomonas reinhardi*, all the enzyme activity was found in the pellet; additionally, the activity of the pellet was no more than that of whole, living cells. Experiments with six other species (Table IV) showed that little of the enzyme activity was in solution either before or after sonication. Here, too, except for *Cricosphaera*, there was no increase in enzyme activity after disruption of the cell structure. In *Coccolithus* the activity of each pH between 7.7 and 9.7 was approximately the same in sonicated as in intact cells. As already discussed, identical rates of assimilation of P from G-6-P,

TABLE IV

Alkaline phosphatase (Units/ml. of culture) in cells and filtrate before and after sonication. The cells were sonicated at 0° C. in 5 N NaCl

	Whole culture		Sonicated culture		
			Cells		Filtrate after
	Cells	Filtrate	Before	After	
Phaeodactylum tricornutum	90.4	9.6	83	80	9.2
Cyclotella cryptica	50	0	52	38	0.1
Chaetoceros simplex	73	0	72	58	0.
Thalassiosira fluviatilis	64.4	1.6	74	57	0.24
Isochrysis galbana	130	0	103	70	0
Cricosphaera carterae	141	0	119	201	0

AMP, and α-GP more likely result from hydrolysis at the cell surface than from identical rates of transport of the whole molecules into the cells. Finally, the nitrophenol resulting from hydrolysis of NPP was found in solution, just as the C^{14} from labeled G-6-P stayed in solution (Fig. 6); these facts are more readily explained by extracellular hydrolysis of non-penetrating substrates than by immediate excretion of nitrophenol, labeled glucose, or labeled CO_2 following intracellular hydrolysis. Our evidence for cell-surface enzymatic activity in algae agrees with that of Brandes and Elston (1956), Talpasayi (1962), Galloway and Krause (1963), and Overbeck (1962) already mentioned in our introduction.

DISCUSSION

It appears that the alkaline phosphatases of marine algae may enable them to regenerate phosphate from organic sources and thus circumvent heterotrophic regeneration. In general, heterotrophs may be expected to hydrolyze phosphate esters in order to obtain the energy bound in the organic moiety, whereas autotrophs with abundant energy available from sunlight would only need the inorganic phosphate radical. There are similarities, however, between phosphatases of yeasts and bacteria and those of algae. Suomalainen *et al.* (1960) reported that both the acid

and alkaline phosphatases of baker's yeast increased markedly with phosphorus starvation. From comparisons of enzyme activity of whole cells to that of dried, freeze-thawed, or cytolyzed cells, they concluded that the acid phosphatase was mostly at the cell surface and the alkaline phosphatase was mostly internal. In the bacterium, *Escherichia coli*, Torriani (1960) found that the acid phosphatase was always present (*i.e.*, constitutive), whereas alkaline phosphatase synthesis was repressed by phosphate. Malamy and Horecker (1961) removed the cell walls from *E. coli* with lysozyme and reported that these protoplasts lost most of their alkaline phosphatase activity. They suggested that the enzyme lies outside the cell membrane. Yeasts and bacteria, therefore, also have a means of hydrolyzing phosphate esters extracellularly, especially when P-deficient.

The ability to produce phosphatases that act on extracellular substrates may give some algae a competitive advantage over other species when phosphate in sea water is less abundant than dissolved organic phosphorus. It is probable that these phosphatases not only hydrolyze but also immediately transfer the phosphate to an acceptor in the cell. Such a transfer would improve the recovery efficiency and increase the competitive advantage even more. The components of the dissolved organic phosphorus pool in the sea are still completely unknown, but undoubtedly some of the compounds are phosphomonoesters. Kuenzler *et al.* (1963) reported that P-deficient *Phaeodactylum tricornutum* could rapidly obtain more phosphorus from natural sea water than was present as orthophosphate, and concluded that the difference came from dissolved organic phosphorus compounds.

We have tried to learn the characteristics of the phosphatases of whole algae and the resulting capabilities for the living cells. In this regard, experiments on enzyme activity at very high or very low pH (Fig. 3) are not very meaningful because such conditions are abnormal in the sea and often lethal in cultures. Sea water usually has a pH of 8 or higher where active photosynthesis is occurring, and it seems significant that the phosphatases synthesized can hydrolyze esters at sea water pH. Constitutive internal phosphatases can be expected to have optima suited to particular pH values of various sites within the cell.

It is possible that the ratio of phosphatase to cell volume, protein, or chlorophyll in natural phytoplankton populations can give direct information about their nutrient status. The fact that phosphate is undetectable in sea water does not prove that the natural phytoplankton population is phosphorus-deficient. The limit of chemical detectability is not yet as low as the limit of assimilability by algae (Kuenzler and Ketchum, 1962). From Tables II and III we would guess that samples of phytoplankton dominated by chrysophytes and diatoms are phosphorus-deficient if the alkaline phosphatase activity is greater than 10×10^{-8} Units/μ^3 of cells, *i.e.*, two days' production of this enzyme at a rate of 5×10^{-8} Units/$\mu^3 \cdot$day. We have been able to detect alkaline phosphatase in the particulate matter of some samples of natural sea water. More analyses must be done, however, before we can draw conclusions about the phosphate nutrition of the plankton from enzyme measurements.

Summary

1. Phosphate-repressible alkaline phosphatases were found in axenic cultures of marine algae, especially Chrysophyceae and Bacillariophyceae. Enzyme synthesis

started when the algae became phosphorus-deficient and stopped if phosphate was restored to the medium.

2. Maximal enzyme activity was usually above pH 9, but significant activity was also present at ordinary sea water pH, approximately pH 8.

3. The phosphatases enabled algae to split glucose-6-phosphate; the phosphate was quickly assimilated but the glucose moiety remained in the medium. Algae took phosphorus from adenosine monophosphate and α-glycerophosphate at the same rate as from glucose-6-phosphate.

4. The phosphatases appear to be firmly bound near the cell surface. They may enable deficient algae to regenerate phosphate from soluble organic phosphorus compounds present in natural sea water.

LITERATURE CITED

Brandes, D., and R. N. Elston, 1956. An electron microscopical study of the histochemical localization of alkaline phosphatase in the cell wall of *Chlorella vulgaris*. *Nature*, **177**: 274–275.

Eppley, R. W., 1962. Hydrolysis of polyphosphates by *Porphyra* and other seaweeds. *Physiol. Plant.*, **15**: 246–251.

Galloway, R. A., and R. W. Krauss, 1963. Utilization of phosphorus sources by *Chlorella*. *In:* Microalgae and Photosynthetic Bacteria; Plant and Cell Physiol. Spec. Issue, pp. 569–575.

Guillard, R. R. L., and J. H. Ryther, 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt, and *Detonula confervacea* (Cleve) Gran. *Canad. J. Microbiol.*, **8**: 229–239.

Ketchum, B. H., N. Corwin and D. J. Keen, 1955. The significance of organic phosphorus determinations in ocean waters. *Deep-Sea Res.*, **2**: 172–181.

Kuenzler, E. J., R. R. L. Guillard and N. Corwin, 1963. Phosphate-free sea water for reagent blanks in chemical analyses. *Deep-Sea Res.*, **10**: 749–755.

Kuenzler, E. J., and B. H. Ketchum, 1962. Rate of phosphorus uptake by *Phaeodactylum tricornutum*. *Biol. Bull.*, **123**: 134–145.

Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall, 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**: 265–275.

Malamy, M., and B. L. Horecker, 1961. The localization of phosphatase in *E. coli* K_{12}. *Biochem. Biophys. Res. Com.*, **5**: 104–108.

Overbeck, J., 1962. Untersuchungen zum Phosphathaushalt von Grünalgen. II. Die Verwertung von Pyrophosphat und organisch gebundenen Phosphaten und ihre Beziehung zu den Phosphatasen von *Scenedesmus quadricauda* (Turp.) Brep. *Arch. Hydrobiol.*, **58**: 281–308.

Price, C. A., 1962. Repression of acid phosphatase synthesis in *Euglena gracilis*. *Science*, **135**: 46.

Provasoli, L., 1958. Nutrition and ecology of protozoa and algae. *Ann. Rev. Microbiol.*, **12**: 279–308.

Rothstein, A., 1954. The enzymology of the cell surface. *In:* Protoplasmatologia II.E.4. (L. V. Heilbrunn and F. Weber, eds.) Springer-Verlag, Wien; pp. 1–86.

Stadtman, T., 1961. Alkaline phosphatases. *In:* The Enzymes (P. D. Boyer, H. Lardy and K. Myrbäck, eds.). Academic Press: New York. Vol. 5, pp. 55–71.

Suomalainen, H., M. Linko and E. Oura, 1960. Changes in the phosphatase activity of baker's yeast during the growth phase and location of the phosphatases in the yeast cell. *Biochim. Biophys. Acta*, **37**: 482–490.

Talpasayi, E. R. S., 1962. Acid phosphatase activity of some algae and its inhibition by molybdenum. *Biochim. Biophys. Acta*, **59**: 710–712.

Torriani, A., 1960. Influence of inorganic phosphate in the formation of phosphatases by *Escherichia coli*. *Biochim. Biophys. Acta*, **38**: 460–469.

Wooster, W. S., and N. W. Rakestraw, 1951. The estimation of dissolved phosphate in sea water. *J. Mar. Res.*, **10**: 91–100.